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# Improvement of Host Response to Sepsis by Photobiomodulation

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***Background and Objective:*** **Late sepsis causes immunosuppression and is associated with energy depletion in lymphocytes. Adjuvant treatment with ATP-$MgCL_2$ appears to improve cellular energetics and decrease mortality. Laser irradiation can promote cell proliferation and increase cellular ATP synthesis, which may improve the host immune response in sepsis. The purpose of this study was to determine whether laser irradiation (LI) has a stimulatory effect on the immune response in sepsis using an animal model.**

***Study Design/Materials and Methods:*** **The cecal ligation and puncture (CLP) rat model was used. Thirty-six SD rats were divided equally among four groups: control (nonoperative), sham operation, CLP treated with laser irradiation, and CLP without laser irradiation. The peritoneal cavity of each animal in CLP/laser group was irradiated immediately after CLP using an Argon-dye laser at a wavelength of 630 nm and at a fluence of 5 J/$cm^2$. Some animals were euthanized 24 hr following CLP and were used to evaluate the immune response (lymphoctye proliferation). In a separate experiment, the survival of septic rats was observed for 60 days. Lymphocytes isolated from normal rat spleens were used to observe for biostimulatory effects in vitro.**

***Results:*** **LI significantly improved ex-vivo lymphoctye proliferation of cells from septic rats (179.7 ± 17.2 vs. 129.5 ± 7.8; $P < 0.01$) and enhanced survival in septic rats (79% vs. 42%; $P < 0.001$). LI significantly stimulated lymphoctye proliferation in the presence of mitogenic stimuli and enhanced lymphoctye ATP synthesis ($P < 0.05$).**

***Conclusion:*** **LI improves the host immune response and survival rate in sepsis in an animal model. Our studies suggest that LI may be useful as an adjuvant therapy for sepsis. Lasers Surg. Med. 21:262–268, 1997.** **© 1997 Wiley-Liss, Inc.**

## INTRODUCTION

Sepsis is a major cause of morbidity and mortality in surgical intensive care units. Although aggressive and judicious use of antibiotics [1], enteral feeding [2], and the maximization of oxygen delivery [3] have provided physicians with an improved armamentarium, sepsis remains a potent instigator of multiple organ system failure. Current evidence suggests that multiple organ system failure in sepsis is associated with the release of various mediators [4]. Although some of these mediators can become toxic, they play a role in the host's defense response by triggering humoral enzymatic mechanisms involving the complement, intrinsic clotting, fibrinolytic, and kinin pathways. The pathophysiology of polybacterial sepsis involves the identification of various mediators or cytokines that are synthesized and are released by macrophages and other mononuclear cells af-

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ter exposure to endotoxin [5]. Early sepsis results in an ill-directed inflammatory burst. Late sepsis seems to result in immunosuppression with a near shutdown of immunoresponsiveness [6]. Decreased concentrations of high energy phosphates have been implicated as being contributory to organ dysfunction following shock. Changes in lymphoctye energetics are associated with the profound immune dysfunction that occurs late in septic shock. Adjuvant treatment with ATP-$MgCl_2$ has significantly improved tissue ATP levels and organ function in late sepsis and hemorrhagic shock, suggesting that the restoration of immune cell energy substrates may be important in the treatment of sepsis, particularly in late sepsis [7,8].

Photo-biostimulation has been used for a variety of medical therapies, such as chronic or inflammatory wounds and pain control as well as for basic research. Photo-irradiation at low fluences can generate significant bio-effects that are manifested in biochemical, physiological and proliferative phenomena in various enzymes, cells, tissues, organs, and organisms. The phenomena of stimulation and inhibition caused by laser light are termed "laser biostimulation" and "bioinhibition." In vitro data demonstrates that laser irradiation (LI) can stimulate cellular proliferation [9–13], collagen synthesis [10,11], and the release of growth factors from cells [12]. Action spectra show peaks in the blue (404 and 454 nm), red (620 nm), and near infra-red (760 and 830 nm) ranges. Most studies suggest that laser biostimulation occurs at fluences between 0.05–10 J/cm$^2$, whereas fluences above 10 J/cm$^2$ have bioinhibitory effects [14–17]. Our previous studies have demonstrated that LI at certain wavelengths and fluences can stimulate cellular proliferation, increase the release of growth factors from cells in vitro, and significantly improve wound healing in genetically diabetic mice [18]. In separate studies, we have observed increased levels of ATP production in 3T3 fibroblasts, human fibroblasts, and dog kidney cells following LI (unpublished). Our results appear to document a significant increase in ATP levels in cells following LI at certain fluences and wavelengths. Based on results of our studies and other investigators' studies [19–22], we hypothesize that LI may have biostimulatory effects on immune cells such as lymphocytes and macrophages by increasing ATP levels, and, therefore, may improve the immune response in late sepsis. This hypothesis is supported by evidence that immunosuppression in late sepsis is associated with the energy depletion of lymphocytes and that adjuvant treatment with ATP-$MgCl_2$ can improve cellular energetics and decrease mortality in these systems [7,8].

## MATERIALS AND METHODS

### Animal Preparation

The cecal ligation and puncture (CLP) rat model was used as the sepsis model. For the in vivo studies, 36 male Sprage-Dawley (SD) rats (250g) were divided equally among four groups: control (nonoperative), sham operation, CLP plus laser irradiation, and CLP without laser irradiation. The CLP procedure is as follows: The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and a 2 cm ventral midline incision was made. The cecum was exposed and ligated just distal to the ileocecal valve using 4-0 silk suture to avoid intestinal obstruction, then punctured twice with an 18-gauge needle, and returned to the abdomen. The incision was closed in two layers with continuous 4-0 nylon suture. The animals received 2 ml/100g body weight of normal saline subcutaneously immediately following the operation. Sham-operated rats were treated the same surgically, but the cecum was neither ligated nor punctured. The open peritoneal cavity of each animal in the CLP/laser group was irradiated immediately after CLP using an Argon-dye laser at a wavelength of 630 nm and at a fluence of 5 J/cm$^2$. The laser spot size (~5 cm) was achieved by placing the fiber optic 20 cm from the anterior abdominal wall and was sufficiently large to cover an area that included at least one-third of surface of the rat liver and the intestines. The animals were euthanized at 24 hours following CLP. Lymphocytes from the spleen of each animal were used for the lymphoctye proliferation assay.

### Lymphocyte Proliferation Assay Ex Vivo

The rat spleens were removed aseptically and placed in separate sterile plastic petri dishes containing cold phosphate-buffered saline (PBS). The splenic tissue was gently ground between frosted microscope slides. A single suspension was collected and centrifuged at 300g for 5 min at 4°C. The pellet was collected and the erythrocytes in the cell suspension were lysed using a hypotonic lysing buffer (ACE buffer, pH 7.4). The lymphoctye suspension was centrifuged repeatedly as described above, and the cells were resuspended

in DMEM medium (GIBCO-BRL) with 10% fetal calf serum (FCS) to yield a final concentration of $1 \times 10^6$ cells/ml. Lymphocytes were placed in 96-well plates at $10^5$ cells per well and cultured in DMEM medium with 10% FCS. Phytohemagglutinin (PHA) was used as the mitogen and added to the wells at concentrations of 10 μg/ml, 5 μg/ml, 2.5 μg/ml, and 0 μg/ml. Following a 24-hour culture period, the (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to evaluate lymphocyte proliferation according to the method as described by Mosmann [23]. Briefly, 10 micromilliliters of MTT solution (5 mg/ml in PBS) was added to each well in 96-well plates. After a 4-hour incubation, the media were removed and 100 μl 0.04 M HCL in absolute isopropanol was added to lyse the cells. Color detection was determined using a microtiter plate reader set at the dual wavelengths of 590 nm and 630 nm. The results were expressed as the percentage of the optical density (OD) for each group with no mitogen (0 μg/ml PHA) added vs. the same groups with PHA added.

### Lymphocyte Proliferation Assay In Vitro

Lymphocytes were isolated from the spleens of normal SD rats to observe whether laser irradiation had a direct effect on lymphocytes. The technique used to isolate lymphocytes was the same as described above. Lymphocytes were resuspended in DMEM medium with 10% FCS to yield a final concentration of $1 \times 10^6$ cells/ml, and were placed either in 24-well plates at a concentration of $10^5$ cells per well or in 96-well plates at a concentration of $4 \times 10^4$ cells per well. After a 2-hour incubation, the cells were washed twice with PBS, which was added to each well (100 μl for 96-well plates and 500 μl for 24 well plates). The cells underwent laser irradiation immediately. Following LI, the cells were replenished with either the standard medium, or medium containing 12 μg/ml LPS, 100 U/ml IFN-γ, 500 U/ml TNF-α, and 25 ng/ml IL-1 as mitogens before being returned to the incubator. Following a 24-hour incubation, the MTT assay was used to measure lymphoctye proliferation using the method described previously. Lymphocytes in the nonirradiation groups were handled in the same manner but did not receive laser irradiation.

### Laser Irradiation

A Lexel Aurora Model 600 Argon Dye laser (Lexel, Fremont, CA) at a wavelength of 630 nm and power density at 20 mW/cm$^2$ (±0.8 mW/cm$^2$) was used for these studies. Laser irradiation at a fluence of 5 J/cm$^2$ (spot size: 5 cm diameter at 20 cm distance from laser fiber tip) was used for the in vivo study of the CLP rats. The irradiation exposure of the peritoneal cavity covered at least one-third of the surface of liver and the intestines. Laser irradiation at fluences of 2.4 and 4.8 J/cm$^2$ (spot size: 6 cm diameter at 30 cm distance from laser fiber tip) was used for the in vitro studies. The fluences and the wavelengths utilized in these experiments were chosen based upon our previous investigations and the work of other investigators.

### Quantitation of Lymphocyte Adenosine Triphosphate

An ATP analysis kit (Sigma, St. Louis, MO) was used to measure the ATP content of lymphocytes from both the CLP and normal rats. Briefly, the culture media were removed from the wells and 1.0 ml of 12% trichloroacetic acid (TCA) was added to each well to precipitate the cellular proteins. Following centrifugation at 3,000g for 5 minutes, clear TCA supernatants were obtained. For the ATP assay, 1 ml of PGA buffered solution, 1.5 ml of distilled water, and 0.5 ml of TCA supernatant were added to the 0.3 mg NADH vial and the initial absorption at 340 nm ($A_{340}$) was obtained. After addition of 0.04 ml of glyceraldehyde phosphate dehydrogenase/phosphoglycerate phosphokinase (GAPD/PGK) enzyme mixture to the cuvet, the final $A_{340}$ was recorded at 5 minutes. The protein content in each well was determined using a Pierce BCA protein assay kit. The ATP concentration was calculated as ($\Delta A_{340}$) × 97.7/protein (mg/dl).

### Survival Rates for Septic Rats

In a separate experiment, the survival rate following CLP with and without laser irradiation was evaluated. The survival rate of the rats was assessed for a period of 60 days following the operative procedure. The CLP procedure and laser irradiation were performed as was described previously.

### Statistical Analysis

The data are presented as the mean ± SEM for lymphoctye proliferation, ATP levels, and nitrite concentrations. Differences in the experimental means were considered significant at the $P < 0.05$ level as was determined by the one-way ANOVA and Tukey's test. Survival data were

**TABLE 1. Ex-vitro Lymphocyte Proliferative Capacity†**

| | Optical density % of non-PHA treated control | | | |
|---|---|---|---|---|
| PHA | Normal | Sham | CLP/laser* | CLP |
| 10 μg/ml | 174.9 ± 11.9 | 161.1 ± 10.7 | 179.7 ± 17.2 | 129.5 ± 7.8** |
| 5 μg/ml | 131.5 ± 10.1 | 144.3 ± 9.5 | 171.8 ± 16.9 | 120.5 ± 8.5** |
| 2.5 μg/ml | 105.7 ± 10.3 | 125.3 ± 9.6 | 137.8 ± 13.8 | 105.6 ± 10.1 |

†Lymphocyte proliferation was determined using MTT assay. Lymphocytes were isolated from normal or experimental rat spleens. Numbers expressed are the percentage of optical density in each group without PHA vs. optical density observed in the same groups with PHA. The results are represented as mean ± SE (N = 9).
*Laser irradiation significantly increases lymphoctye proliferation ($P < 0.01$) as compared to CLP.
**CLP (sepsis) group demonstrates depressed lymphoctye proliferation ($P < 0.01$) as compared to control at PHA concentrations of 10 μg/ml and 5 μg/ml.

analyzed via the Chi-square analysis. Differences were considered significant at the $P < 0.05$ level.

## RESULTS

### Lymphocyte Proliferative Capacity Following CLP Procedures

CLP-sepsis caused a significant decrease in lymphoctye proliferative capacity in response to mitogenic stimulation. Lymphocyte proliferative capacity was significantly decreased following CLP (see Table 1). The CLP (sepsis) group demonstrated a depressed lymphoctye response to mitogen (10 μg/ml and 5 μg/ml of PHA) as compared to the control group ($P < 0.01$). Lymphocytes from rats receiving laser irradiation following CLP demonstrated a significantly greater proliferative capacity ($P < 0.01$) as compared to the CLP group without LI.

### Lymphocyte Proliferative Capacity In Vitro

Following laser irradiation, lymphocytes appeared to be hyperresponsive to mixed mitogens. Mixed mitogens had a significantly stimulatory effect on lymphocyte proliferation (Table 2). Following LI at fluences of 2.4 J/cm$^2$ and 4.8 J/cm$^2$, lymphocytes in the presence of mitogens significantly increased cellular proliferation as compared to those lymphocytes that did not receive LI ($P < 0.01$). However, in the absence of mitogens, laser irradiation alone did not affect lymphoctye proliferation.

### ATP Levels of Lymphocytes In Vitro

Laser irradiation had a significantly stimulatory effect on the cellular ATP content of lymphocytes (Table 2). Although mixed mitogens alone can enhance ATP levels of lymphocytes, lymphocytes following laser irradiation at fluences of 2.4 J/cm$^2$ and 4.8 J/cm$^2$ appear to have higher ATP levels than the nonirradiation group ($P < 0.05$). In the absence of mitogens, LI significantly increases the ATP levels when compared with the nonirradiation group ($P < 0.01$).

### Survival Rates Following CLP Sepsis

Overall survival following CLP in rats treated with and without LI is shown in Figure 1. Treatment with laser irradiation following CLP significantly decreased the lethality from sepsis with 42% of animals in the CLP alone group surviving at day 5 as compared to 79% in the CLP plus laser irradiation group ($P < 0.001$). After day 5, no further mortality was observed through 60 days posttreatment.

## DISCUSSION

Sepsis is a risk factor of the development of multiple organ failure and is associated with a high mortality rate. Despite significant advances in defining the complex pathophysiology of sepsis, the mechanisms of the host's cellular responses remain unclear. An early inflammatory burst, which sets off a cascade of detrimental concomitant effects, is associated with a delayed immunologic hypofunction, which seems to fail to effectively eradicate micro-organisms in late sepsis [24–26]. When the host is overwhelmed by a polymicrobial challenge, the immune cells are unable to elaborate immunoresponsive agents. During both late septic shock and hemorrhagic shock, oxygen delivery is decreased. An inadequate supply of oxygen would be expected to deplete cellular energy stores and have a deleterious effect on ATP-dependent cellular functions such as lymphokine synthesis, macrophage cytotoxicity, and antigen presentation [7,8]. The profound immu-

**TABLE 2. Lymphocyte Proliferation and ATP Products In Vitro Following Laser Irradiation**

| Fluences ($J/cm^2$) | Optical density % of nonmitogen control[a] | | ATP (μmol/mg protein)[b] | |
|---|---|---|---|---|
| | Mitogens | No mitogens | Mitogens | No mitogens |
| 0 | 125 ± 2.53 | 100 ± 1.36 | 1.26 ± 0.26 | 0.39 ± 0.068 |
| 2.4 | 150 ± 7.07* | 108 ± 2.86 | 2.23 ± 0.51** | 0.58 ± 0.035*** |
| 4.8 | 147 ± 3.47* | 95 ± 2.73 | 2.01 ± 0.31** | 0.92 ± 0.04*** |

[a]Lymphocyte proliferation was measured using the MTT assay. Numbers are expressed as the percentage of optical density in the experimental groups vs. the optical density observed in the control group not treated with mitogens. The results are represented as the mean ± SE (N = 18). The data were collected from three repeated experiments. The absolutely optical density of MTT assay may vary in different experiments.
[b]ATP production was measured using an enzymatic method. Numbers are represented as ATP (μmol) per mg protein. Results are expressed as the mean ± SEM, N = 8.
*Laser irradiation significantly increases lymphoctye proliferation in the presence of the mitogens ($P < 0.01$) as compared to lymphocytes that did not receive laser irradiation.
**$P < 0.05$ vs. the control in the presence of mitogens.
***$P < 0.05$ vs. the control in the absence of mitogens. The experiments were performed in duplicate.

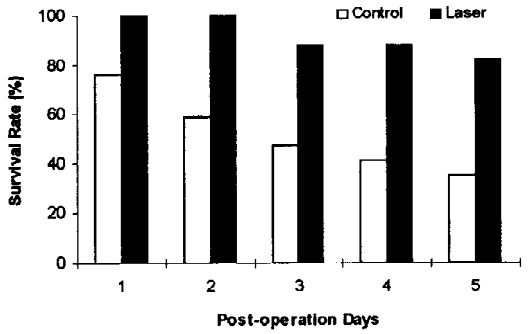


Fig. 1. Survival rates in CLP sepsis rats. Thirty-four rats were equally divided into two groups. Following the CLP, the animals in laser group were treated with laser irradiation as described in Materials and Methods. The control group was treated similarly except that no laser irradiation was given. Survival rate was observed over 60 days. After day 5, no further mortality was observed in either groups. CLP sepsis rats exhibited a significantly enhanced survival rate following laser irradiation as compared to the control animals ($P < 0.001$).

nosuppression in hemorrhagic and late septic shock has been explained in part by decreased ATP levels in macrophages and lymphocytes [8,27]. In this regard, we hypothesized that LI may raise ATP levels in a variety of cells, including lymphocytes and macrophages. Therefore, LI may protect rats challenged with CLP-induced sepsis. Our results indicate that lymphocytes from rats that underwent CLP are indeed hyporesponsive to mitogens. However, the lymphocytes from CLP rats receiving LI had a significantly increased cellular response to mitogenic stimulation as compared to the CLP alone group. Since basic immune cellular energy-dependent processes are depressed following shock [6,7], improvement of the immune response following LI suggests that laser irradiation may stimulate the synthesis of cellular energy and restore cellular and tissue ATP levels. This photo-stimulatory effect may involve the mitochondria as the photoacceptors for visible light energy. The absorption of light by cytochrome *b* and *c* in the respiratory chain may cause a short-term activation of the respiratory chain as well as a secondary dark reaction. This would lead to an increase in the redox reaction of the electron-transport chain. Activation of the electron-transport chain results in an increase of promotive force, an increase in the electrical potential of the mitochondrial membrane and the ATP pool, the alkalization of cytoplasm, and finally, the activation of nucleic acid synthesis and cellular proliferation [14,28].

Our study shows that lymphocytes without mitogen activation following laser irradiation did not increase cellular proliferation. However, laser irradiation did increase the ATP levels in lymphocytes. Lymphocytes isolated from the normal rat spleens were significantly responsive to mitogenic stimulation, thereby causing increases in both lymphoctye proliferation and cellular ATP levels. Lymphocytes were hyperresponsive to mitogens following laser irradiation and produced significantly increased cellular ATP levels as compared to nonirradiated cells. These results indicate that laser irradiation alone does not activate lymphocytes. However, nonactivated lymphocytes may increase ATP production following photoirradiation. Photons from laser irradiation may serve to

accelerate the redox reaction of the electron transport chain. We are uncertain whether there is a limitation on this stimulatory effect in the nonactivated lymphocytes. In the presence of mitogens, activated lymphocytes may require more energy (i.e., ATP) to meet the demands of increased cell proliferation. A remarkable enhancement of ATP production following laser irradiation may provide sufficient energy for cells to respond to mitogenic stimuli and therefore increase cell proliferation.

The significantly increased survival in septic animals in the laser treatment group suggests that LI can protect animals from bacterial challenge secondary to CLP-induced sepsis. Two possible mechanisms may explain these stimulatory effects. First, laser irradiation may accelerate cellular oxidative metabolism and therefore increase free radicals such as nitric oxide and superoxide anion. Our recent studies have found that laser irradiation can significantly increase oxidative metabolism in human neutrophils (unpublished). This could lead to an enhanced antimicrobiral potential in the septic host, to reduce inflammatory responses in the late stage of sepsis, and may be of potential benefit immediately following CLP. Second, laser irradiation may increase ATP production in immune cells and restore the energy depletion that ordinarily occurs during sepsis. This restorative effect may be an immediate response following laser irradiation, in addition to secondary dark reactions.

Although the precise mechanism of the immunoprotective effects of LI is unclear, it is apparent that LI restores splenocyte ATP levels, enhances lymphoctye proliferation in the presence of mitogenic stimuli, and significantly improves survival rates of the treated animals. Our studies suggest that LI may be useful as an adjuvant therapy for sepsis. Our present study examined the splenocyte proliferative capacity. Whether LI also improves the function of other types of immune cells, particularly in vivo, warrants further study.

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